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Echinacoside alleviated LPS-induced cell apoptosis and inflammation in rat intestine epithelial cells by inhibiting the mTOR/STAT3 pathway

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ABSTRACT

Inflammatory bowel disease (IBD) is a chronic and progressive inflammatory condition of colon and small intestine. Echinacoside (ECH) is a phenylethanoid glycoside that possesses various activities, including anti-inflammatory effect. However, the role of ECH in IBD is unknown. The present study aimed to evaluate the effect of ECH on LPS-induced rat intestine epithelial cells and the potential mechanisms. The results showed that LPS inhibited cell viability in time- and dose-dependent manners. ECH treatment attenuated the inhibition effect of LPS on cell viability. ECH alleviated LPS-induced apoptosis of rat intestine epithelial cells. ECH attenuated LPS-induced secretion and mRNA expression of TNF-α and IL-6, but enhanced LPS-induced secretion and mRNA expression of IL-10 and TGF-β1 in IEC-6 cells. The mTOR/STAT3 pathway was activated by LPS, while the activation was inhibited by ECH. Rapamycin, an inhibitor of mTOR, reversed the effect of LPS on rat intestine epithelial cells. In summary, this work suggested that ECH attenuated LPS-induced inflammation and apoptosis in rat intestine epithelial cells via suppressing the mTOR/STAT3 pathway. The findings indicated that ECH might be considered as a potential strategy for the treatment of IBD.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic and progressive inflammatory condition of the colon and small intestine [1,2]. Crohn's disease (CD) and ulcerative colitis (UC) are the two principal types of IBD [3]. There are various clinical symptoms of IBD, such as abdominal pain, vomiting, diarrhea, rectal bleeding and anemia [3,4]. IBD is characterized by increased cytokine production and increased apoptosis of intestinal cells, and thus results in the destruction of intestinal epithelial barrier [5]. Therefore, inhibiting inflammatory response and cell apoptosis might be useful for protecting the intestinal epithelial barrier and be beneficial for IBD treatment.

IBD is characterized by a chronic idiopathic inflammation in intestine, and many pathways associated with inflammatory response are involved in the development and progress of IBD, such as mechanistic target of rapamycin (mTOR) [6] and activator of transcription (STAT) signaling pathways [7]. It was reported that the mTOR/STAT3 pathway plays important roles in inflammation and cell apoptosis [8]. On the other hand, the mTOR/STAT3 signaling was abnormally up-regulated in the colonic epithelial cells of patients with IBD [9], and inhibition the mTOR/STAT3 pathway ameliorated IBD in a mouse model of inflammatory bowel disease [10]. Therefore, the mTOR/STAT3 pathway is a potential therapeutic target for the treatment of IBD.

Echinacoside (ECH, Fig.1) is a phenylethanoid glycoside isolated from *Cistanche* herba, which is usually used for the treatment of constipation in China [11]. It has been reported that ECH possesses various activities, including anti-oxidative [12], anti-inflammatory [13], anti-tumor effect [14]. ECH stimulates cell proliferation and reduces apoptosis of intestinal epithelial cells, indicating that ECH might be useful for the treatment of digestive tract diseases [15]. However, the role of ECH in IBD and the potential mechanism remain unknown.

In the present study, we investigated the role of ECH in LPS-induced intestinal cells and we hypothesized that ECH inhibited LPS-induced inflammation and cell apoptosis via the mTOR/STAT3 pathway.

2. Materials and methods

2.1. Reagents

ECH (purity > 98%) was obtained from Sigma (St. Louis, MO, USA). Rapamycin was purchased from Sigma and dissolved in dimethyl sulphoxide (DMSO; Sigma). Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). The caspase-3 activity assay kit was purchased from Beyotime Biotechnology

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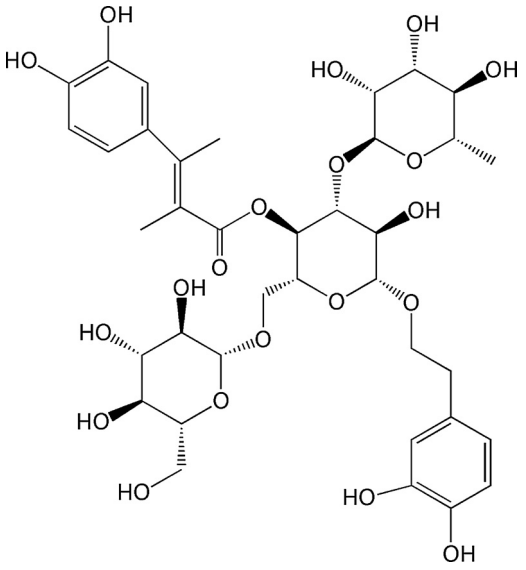

Fig. 1. The chemical structure of ECH.

(Shanghai, China). The enhanced BCA protein assay kit was purchased from Beyotime Biotechnology. The primary antibodies against phospho-mTOR (p-mTOR; Ser2448) and mTOR were obtained from Abcam (Cambridge, MA, USA). The primary antibodies against phospho-ribosomal protein S6 (p-S6; Ser235/236), ribosomal protein S6 (S6), phospho-STAT3 (p-STAT3; Tyr705), STAT3 and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). The secondary antibody was obtained from Cell Signaling Technology. The enhanced chemiluminescence system was purchased from GE healthcare (CA, USA). The ELISA kits for determination of TNF-α, IL-6, IL-10, and TGF-β1 were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Cell culture

Rat intestinal cell line IEC-6 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). IEC-6 cells were cultured in DMEM supplemented with 10% (v:v) FBS, 10 U/ml insulin, and 100 U/ml penicillin and streptomycin, and maintained at 37 °C in a 5% CO_2 environment.

2.3. MTT assay

Cell viability of IEC-6 cells was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The IEC-6 cells (1×10^5/ml) were seeded into a 96-well plate and incubated under different conditions. After incubation, 20 μl MTT solution (5.0 mg/ml) was added and incubated for 4 h. The formazan crystals were dissolved by adding 150 μl DMSO. Finally, the absorbance at 490 nm was measured using an EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). The experiments were repeated thrice in three replicates.

2.4. Flow cytometry

The apoptosis rate of IEC-6 cells was detected by flow cytometry. After treatment with ECH (10 or 20 μg/ml) or rapamycin (100 nM) in the presence of LPS (l μg/ml) for 24 h, cells were collected and stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) for 15 min at room temperature in the dark. Finally, cells were analyzed by flow cytometry (BD Biosciences) according to the manufacturer's instructions. The experiments were repeated three times.

2.5. Detection of caspase-3 activity

The activity of caspase-3 was measured by caspase-3 activity assay kit according to the manufacturer's instruction. Briefly, cells were collected and lysed, and the substrate Ac-DEVD-pNA (2 mM) was added and incubated at 37 °C for 2 h. Finally, the absorbance at 405 nm was measured using an EnSpire Multimode Plate Reader. The experiments were repeated three times.

2.6. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from IEC-6 cells with Trizol reagent (Invitrogen). and used for first-strand cDNA synthesis by reverse transcription with a Transcriptor First Strand cDNA Synthesis Kit (Takara, Dalian, China). The gene expression was tested by qRT-PCR using SYBR PremeScript miRNA RT-PCR kit (Takara). The expression of mRNA was normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers were as follows: TNF-α, forward 5'-ACTG AACT TCGG GGTG ATTG-3', reverse 5'-GCTT GGTG GTTT GCTA CGAC-3'; IL-6, forward 5'-GGCT AAGG ACCA AGAC CATC CAA-3', reverse 5'-TCTG ACCA CAGT GAGG AATG TCCA-3'; IL-10, forward 5'-GTTG CCAA GCCT TGTC AGAA A-3', reverse 5'-TTTC TGGG CCAT GGTT CTCT-3'; TGF-β1, forward 5'-GACC GCAA CAAC GCAA TCTA-3', reverse 5'-AGGT GTTG AGCC CTTT CCA-3'; GAPDH, forward 5'-TGAA GCAG GCAT CTGA GGG-3', reverse 5'-CGAA GGTG GAAG AGTG GGAG-3'. Relative expression levels of target mRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method. The experiments were repeated three times.

2.7. Western blot

After treatment with ECH (10 or 20 μg/ml) or rapamycin (100 nM) in the presence of LPS (l μg/ml) for 24 h, cells were lysed using radioimmunoprecipitation assay buffer (Beyotime). The protein concentration was measured using BCA assay. Protein samples were separated using 12% SDS-PAGE gel and transferred onto nitrocellulose membranes. Then the non-specific bindings were blocked using 5% nonfat milk at 37 °C for 1 h. The membranes were incubated with primary antibodies against p-mTOR (Ser2448), p-S6 (Ser235/236), S6, mTOR, p-STAT3 (Tyr705), STAT3 and β-actin at 4 °C overnight, and then incubated with peroxidase-conjugated secondary antibody at 37 °C for 1 h. Finally, the bands were visualized using the enhanced chemiluminescence kit. The experiments were repeated three times.

2.8. ELISA

After treatment with ECH (10 or 20 μg/ml) or rapamycin (100 nM) in the presence of LPS (l μg/ml) for 24 h, cell culture supernatants were collected for determination of pro-inflammatory and anti-inflammatory cytokine secretion. The secretion levels of TNF-α, IL-6, IL-10, and TGF-β1 from IEC-6 cells were measured using commercial ELISA kits. The experiments were repeated thrice in three replicates.

2.9. Statistical analysis

Data are presented as mean ± standard deviation (SD). Data analysis was examined by one-way ANOVA or Student's *t* test using SPSS version 19.0. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. LPS suppressed cell viability of IEC-6 cells

To evaluate the effect of LPS on cell viability, IEC-6 cells were incubated with LPS at a series of concentrations (0, 0.25, 0.5, 1, 2, and 4 μg/ml) for 24 h. The results in Fig. 2A showed that LPS inhibited cell

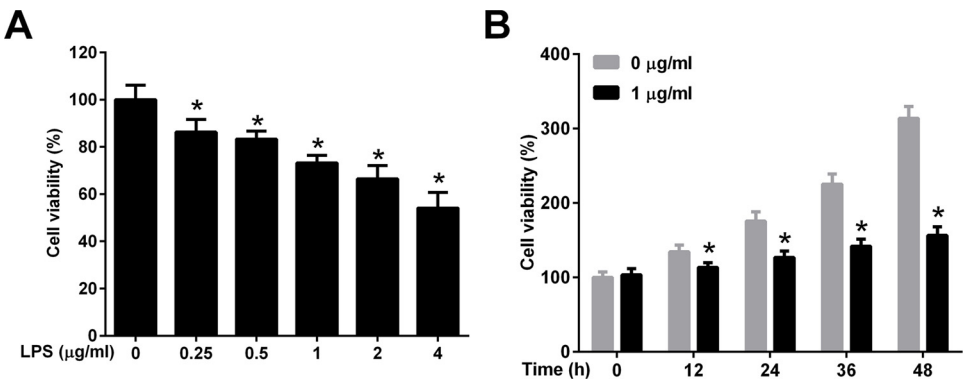

Fig. 2. LPS suppressed cell viability of IEC-6 cells. MTT assay was performed to evaluate the effect of LPS on cell viability. (A) IEC-6 cells were treated with LPS at a series of concentrations (0, 0.25, 0.5, 1, 2, and 4 μg/ml) for 24 h. (B) IEC-6 cells were treated with or without l μg/ml of LPS for a series of time (0, 12, 24, 36, and 48 h). *$p < 0.05$ vs. cells without LPS treatment. Significance was determined by one-way ANOVA.

viability in a dose-dependent manner. In addition, IEC-6 cells were incubated with or without l μg/ml of LPS for a series of time (0, 12, 24, 36, and 48 h). As shown in Fig. 2B, LPS inhibited cell viability in a time-dependent manner.

3.2. ECH increased cell viability of IEC-6 cells

To evaluate the cytotoxicity effect of ECH on IEC-6 cells, cells were incubated with ECH (0, 5, 10, 20, and 30 μg/ml) for 24 h. The results in Fig. 3A indicated that the cell viability was not significantly affected by ECH, suggesting that ECH exhibited nontoxic effect on IEC-6 cells. To further investigated the protective effect of ECH on LPS-induced IEC-6 cells, cells were treated with ECH (10 or 20 μg/ml) and LPS (l μg/ml). As shown in Fig. 3B, ECH treatment (10 or 20 μg/ml) attenuated the inhibition effect of LPS on cell viability.

3.3. ECH inhibited LPS-induced apoptosis of IEC-6 cells

To evaluate the effect of ECH on LPS-induced apoptosis of IEC-6 cells, the apoptosis rate was detected by flow cytometry. LPS induced apoptosis of IEC-6 cells, and ECH treatment (10 or 20 μg/ml) attenuated the induction (Fig. 4A). We also determined the caspase-3 activity. As shown in Fig. 4B, the caspase-3 activity was increased in cells treated with LPS. However, the caspase-3 activity was decreased in cells treated with LPS and ECH, compared with cells treated with LPS. In addition, the expression levels of Bcl-2 and Bax were detected by western blot. The results in Fig. 4C showed that LPS inhibited Bcl-2 expression and induced Bax expression in IEC-6 cells. ECH attenuated the effect of LPS on expression of Bcl-2 and Bax. The findings indicated that ECH inhibited LPS-induced apoptosis of IEC-6 cells.

3.4. ECH inhibited LPS-induced inflammation

TNF-α and IL-6 are two important pro-inflammatory cytokines that are significantly increased in IBD [16]. IL-10 and TGF-β1 are two anti-inflammatory cytokines that are involved in IBD [17]. The secretion of TNF-α, IL-6, IL-10, and TGF-β1 was measured by ELISA. As shown in Fig. 5A-D, LPS significantly induced secretion of TNF-α, IL-6, IL-10, and TGF-β1 in IEC-6 cells. ECH treatment (10 or 20 μg/ml) attenuated the induction effect of LPS on production of TNF-α and IL-6, but enhanced LPS-induced the production of IL-10 and TGF-β1 in IEC-6 cells. qRT-PCR was used to detected mRNA levels of TNF-α, IL-6, IL-10, and TGF-β1 in IEC-6 cells. As shown in Fig. 5E-H, LPS increased the levels of TNF-α, IL-6, IL-10, and TGF-β1 mRNA in IEC-6 cells, but ECH treatment (10 or 20 μg/ml) attenuated LPS-induced TNF-α and IL-6 mRNA levels. ECH treatment (10 or 20 μg/ml) enhanced LPS-mediated increase in the levels of IL-10 and TGF-β1 mRNA in IEC-6 cells. These findings suggested that ECH inhibited LPS-induced inflammation in IEC-6 cells.

3.5. ECH inhibited LPS-induced phosphorylation of mTOR and STAT3

It has been reported that the mTOR/STAT3 pathway plays an important role in IBD, and we speculated that it might participate in the effect of ECH. The expression of mTOR, p-mTOR, S6, p-S6, STAT3, and p-STAT3 was detected by western blot. As shown in Fig. 6, we found that LPS exhibited an obvious induction in the expression of p-mTOR, p-S6, and p-STAT3. ECH treatment (10 or 20 μg/ml) inhibited the induction of LPS on p-mTOR, p-S6, and p-STAT3 expression. However, the expression of mTOR, S6, and STAT3 did not affected by either LPS or ECH, indicating that ECH inhibited the mTOR/STAT3 pathway in IEC-6 cells.

3.6. Inhibition of mTOR/STAT3 pathway restored the effects of LPS on IEC-6 cells

To further confirm the role of the mTOR/STAT3 pathway in the effect of LPS, the inhibitor of mTOR, rapamycin, was used. As shown in Fig. 7A, rapamycin treatment alone did not affect the cell viability,

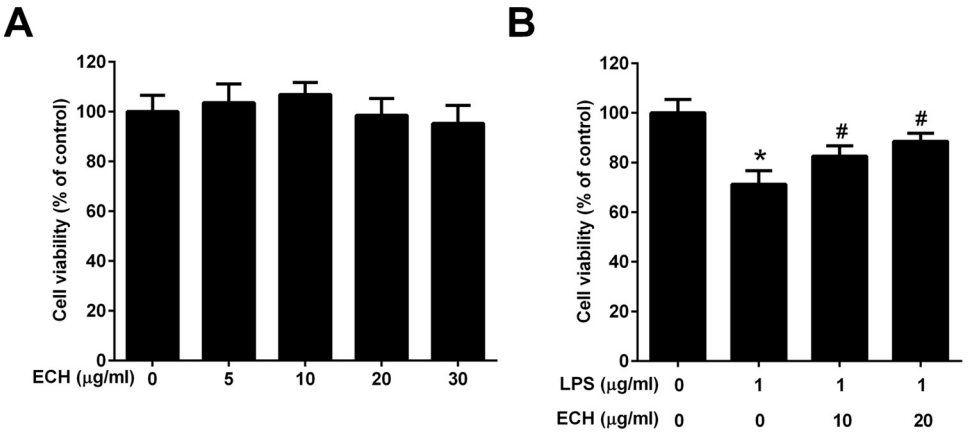

Fig. 3. ECH increased cell viability of IEC-6 cells. MTT assay was performed to evaluate the effect of ECH on cell viability. (A) IEC-6 cells were incubated with ECH (0, 5, 10, 20, and 30 μg/ml) for 24 h. (B) IEC-6 cells were treated with ECH (10 or 20 μg/ml) and LPS (l μg/ml) for 24 h. *$p < 0.05$ vs. control cells (without treatment of LPS and ECH). #$p < 0.05$ vs. cells treated with LPS only. Significance was determined by one-way ANOVA.

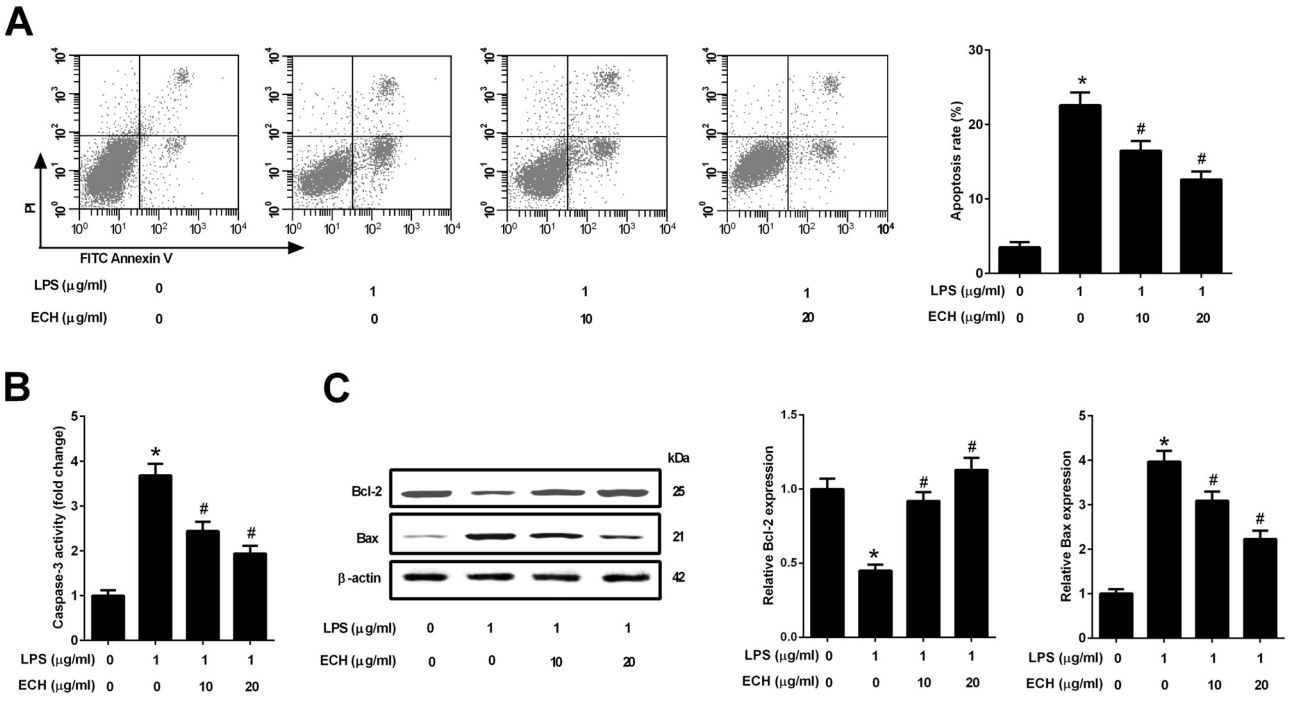

Fig. 4. ECH inhibited LPS-induced apoptosis of IEC-6 cells. IEC-6 cells were treated with ECH (10 or 20 μg/ml) and LPS (l μg/ml) for 24 h. (A) The apoptosis rate was detected by flow cytometry. The sum of the percentages of early apoptosis (lower right quadrant) and late apoptosis (upper right quadrant) is presented as percentages of apoptosis rata in the histogram. (B) Determination of the caspase-3 activity. (C) The expression levels of Bcl-2 and Bax were detected by western blot. $^{*}p < 0.05$ vs. control cells (without treatment of LPS and ECH). $^{\#}p < 0.05$ vs. cells treated with LPS only. Significance was determined by one-way ANOVA.

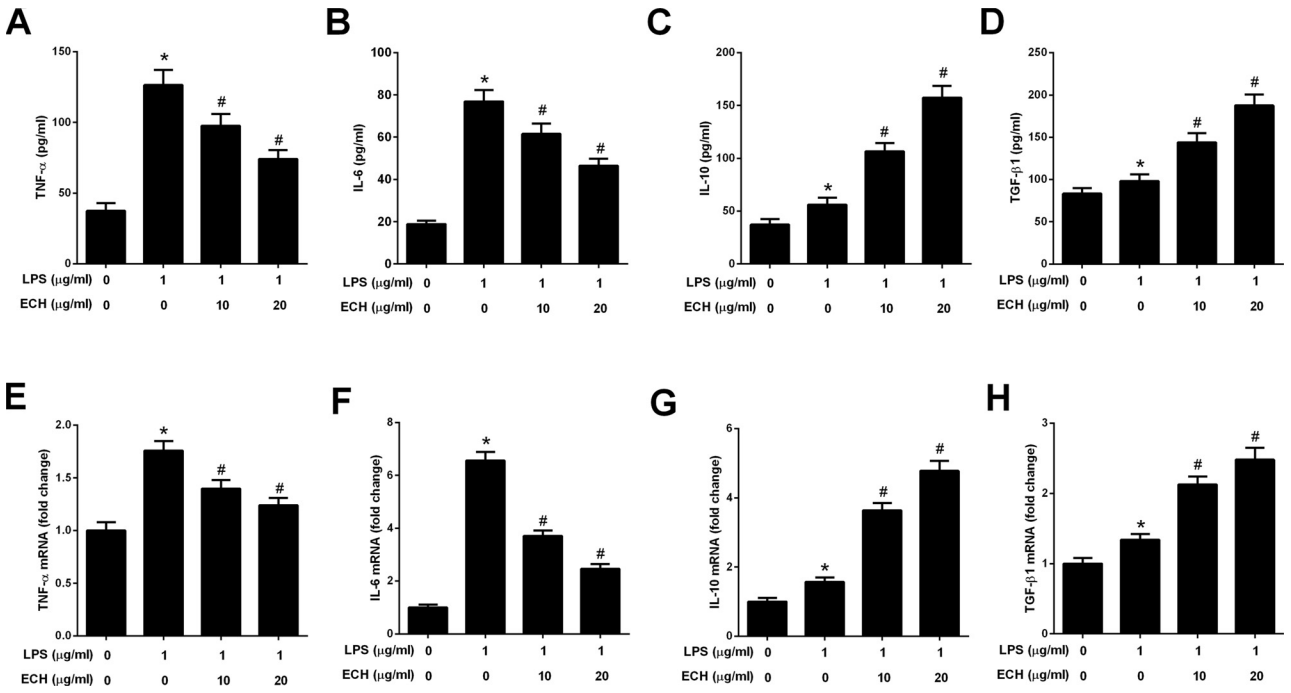

Fig. 5. ECH repressed LPS-induced inflammation. IEC-6 cells were treated with ECH (10 or 20 μg/ml) and LPS (l μg/ml) for 24 h. The secretion of TNF-α (A), IL-6 (B), IL-10 (C), and TGF-β1 (D) from IEC-6 cells was measured by ELISA. The mRNA levels of TNF-α (E), IL-6 (F), IL-10 (G), and TGF-β1 (H) in IEC-6 cells were determined by qRT-PCR. $^{*}p < 0.05$ vs. control cells (without treatment of LPS and ECH). $^{\#}p < 0.05$ vs. cells treated with LPS only. Significance was determined by one-way ANOVA.

however, rapamycin treatment increased the cell viability in LPS-treated cells. Rapamycin treatment alone did not affect the apoptosis rate, caspase-3 activity, and the expression of Bcl-2 and Bax, while rapamycin reduced the apoptosis rate, caspase-3 activity and Bax expression, and induced Bcl-2 expression in cells treated with LPS (Fig. 7B-D). Rapamycin inhibited the LPS-mediated secretion of TNF-α and IL-6, but enhanced LPS-mediated secretion of IL-10 and TGF-β1 (Fig. 7E). In addition, treatment with rapamycin suppressed the expression of p-STAT3 in the presence of LPS (Fig. 7F). Taken together, these data suggested that inhibition of the mTOR/STAT3 pathway

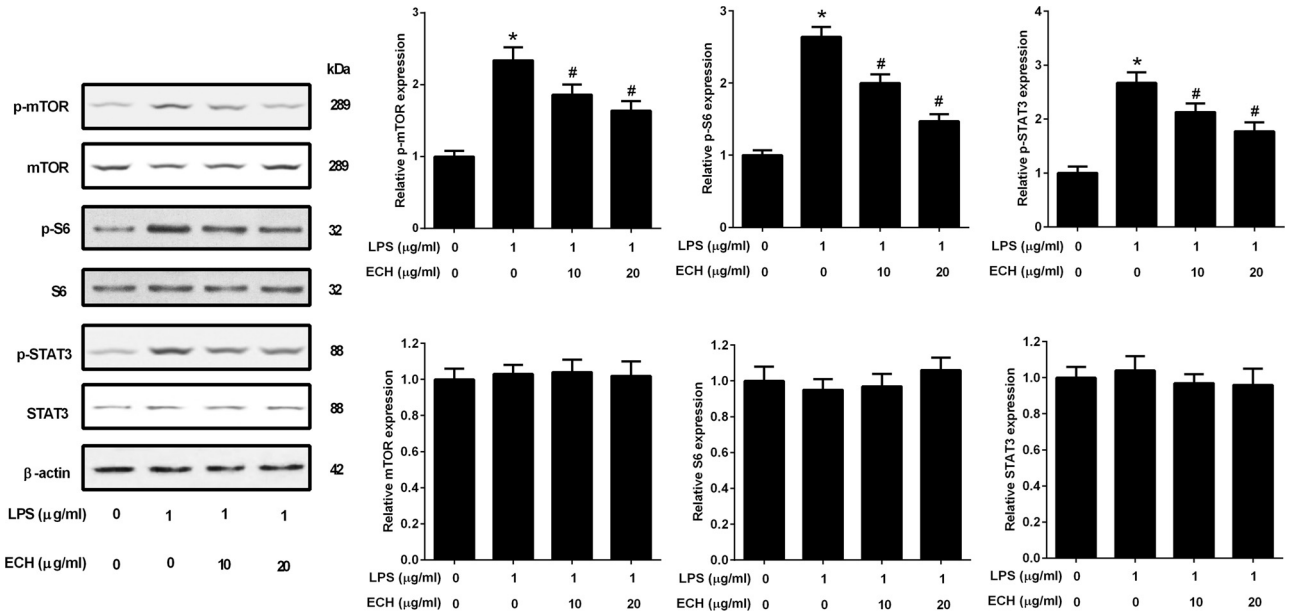

Fig. 6. ECH inhibited LPS-induced phosphorylation of mTOR and STAT3. IEC-6 cells were treated with ECH (10 or 20 μg/ml) and LPS (l μg/ml) for 24 h. The expression of mTOR, p-mTOR, S6, p-S6, STAT3, and p-STAT3 was detected by western blot. $^{*}p < 0.05$ vs. control cells (without treatment of LPS and ECH). $^{\#}p < 0.05$ vs. cells treated with LPS only. Significance was determined by one-way ANOVA.

reversed the effects of LPS on IEC-6 cells.

4. Discussion

Cell death mechanisms have been proved to be associated with the development of IBD [18]. High levels of apoptosis rate have been observed in the intestinal epithelium of patients with IBD [19]. Increasing evidence showed that apoptosis could serve as a therapeutic paradigm in IBD [20]. Runt-related transcription factor 2 (Runx2) inhibited TNF-α-induced apoptosis of colon epithelial cells via down-regulating the expression levels of Bax and cleaved caspase-3, indicating Runx2 possessed protective role in IBD through inhibiting cell apoptosis [21]. Multiple microRNAs are found to be aberrant expressed and play important role in IBD, indicating that microRNAs might be potential target for IBD therapy [22]. MicroRNA-665 (miR-665) was markedly upregulated in active colitis [23]. Ectopic expression of miR-665 promoted cell apoptosis under different inflammatory stimuli [23]. An in vivo study also demonstrated that injection of antagomiR-665 markedly attenuated dextran sulfate sodium-induced apoptosis and colitis [23]. However, the molecular target therapy is complicated.

Recently, many kinds of natural compounds have been showed to possess anti-apoptosis effect. ECH is a phenylethanoid glycoside and has been reported to have anti-apoptosis activity [24]. In an in vivo study, ECH protects mice from LPS-induced acute liver injury via inhibiting hepatocyte apoptosis [24]. ECH suppresses 1-methyl-4-phenylpyridinium ion (MPP +)-induced expression of apoptotic genes including activating transcription factor 3 (ATF3), C/EBP-homologous protein (CHOP), and synuclein alpha (SCNA) in neuroblastoma cells [25]. ECH also markedly decreased MPP +-induced caspase-3 activity in a dose-dependent manner [25]. A previous study showed that ECH-enriched extract of *Cistanche tubulosa* suppressed the development of acute colitis in a preclinical mouse model of ulcerative colitis, suggesting the potential of ECH for treating IBD [26]. In addition, ECH stimulated cell proliferation and enhanced cell survival by reducing cell apoptosis in an intestinal epithelial cell line [15]. In the present study, we also found that ECH promoted cell viability and inhibited cell apoptosis in LPS-induced cells.

Cytokines play an important role in the pathogenesis of IBD. It has been demonstrated that therapy based on cytokines and anti-cytokine antibodies is a promising method for IBD treatment. TNF-α and IL-6 are two kinds of pro-inflammatory cytokines which are significantly increased in IBD [16]. Clinical studies have shown that administration of anti-TNF-α therapy exhibits an important improvement in patients with CD [27]. It was reported that LPS induced strong inflammatory response in an acute liver injury mouse model and ECH exhibited a significant reduction in the inflammatory markers (including nucleosomes and high-mobility group box 1) and inflammatory cytokines in the plasma of acute liver injury mice [24]. ECH could significantly reduce the release of IL-1β and IL-6 which is induced by 6-Hydroxydopamine (6-OHDA) in PC12 cells [13]. By intraperitoneal injection of astilbin, the severity of colitis was alleviated, and the serum levels of IL-10 and TGF-β were increased in dextran sulfate sodium-induced acute colitis in mice [28]. In our work, we used LPS to induce inflammatory response in vitro. We found that ECH attenuated LPS-induced secretion and mRNA expression of TNF-α and IL-6, but enhanced LPS-induced secretion and mRNA expression of IL-10 and TGF-β1 in IEC-6 cells.

STAT3 is a transcription factor belonging to STAT protein family [29]. STAT3 is phosphorylated by JAK in response to cytokines [29]. It has been proved that hyperactivation of STAT3 results in severe colitis, and downregulating STAT3 activity plays an important role for controlling colitis [30]. STAT3 phosphorylation is increased in colon tissues from pediatric patients with active UC [31]. Increased expression and activity of STAT3 could promote inflammation and pathogenesis of UC in children [31]. The inhibition of the JAK/STAT pathway was considered as a critical mechanism by which luteolin showed its anti-inflammatory effect in a cellular model of intestinal inflammation [32]. In our work, we also found that STAT3 expression was increased by LPS and was inhibited by ECH. However, several studies reported positive effect of STAT3 in IBD. Willson et al. [33] reported that loss of intestinal epithelial STAT3 led to more severe chronic inflammation following acute injury. Interleukin-11 reduces TLR4-induced colitis and restores STAT3 pathway in TLR2-deficient mice. Similarly, it has been reported that STAT3 has an oncogenic or a tumor suppressor role depending upon the mutational background of the tumor [34]. Both hyperactivation and insufficient of STAT3 can contribute to the development of IBD. Therefore, the controversial findings might be because the intracellular environment is different, and the levels of STAT3 activity are different. STAT3 has been shown to interact with many molecules, such

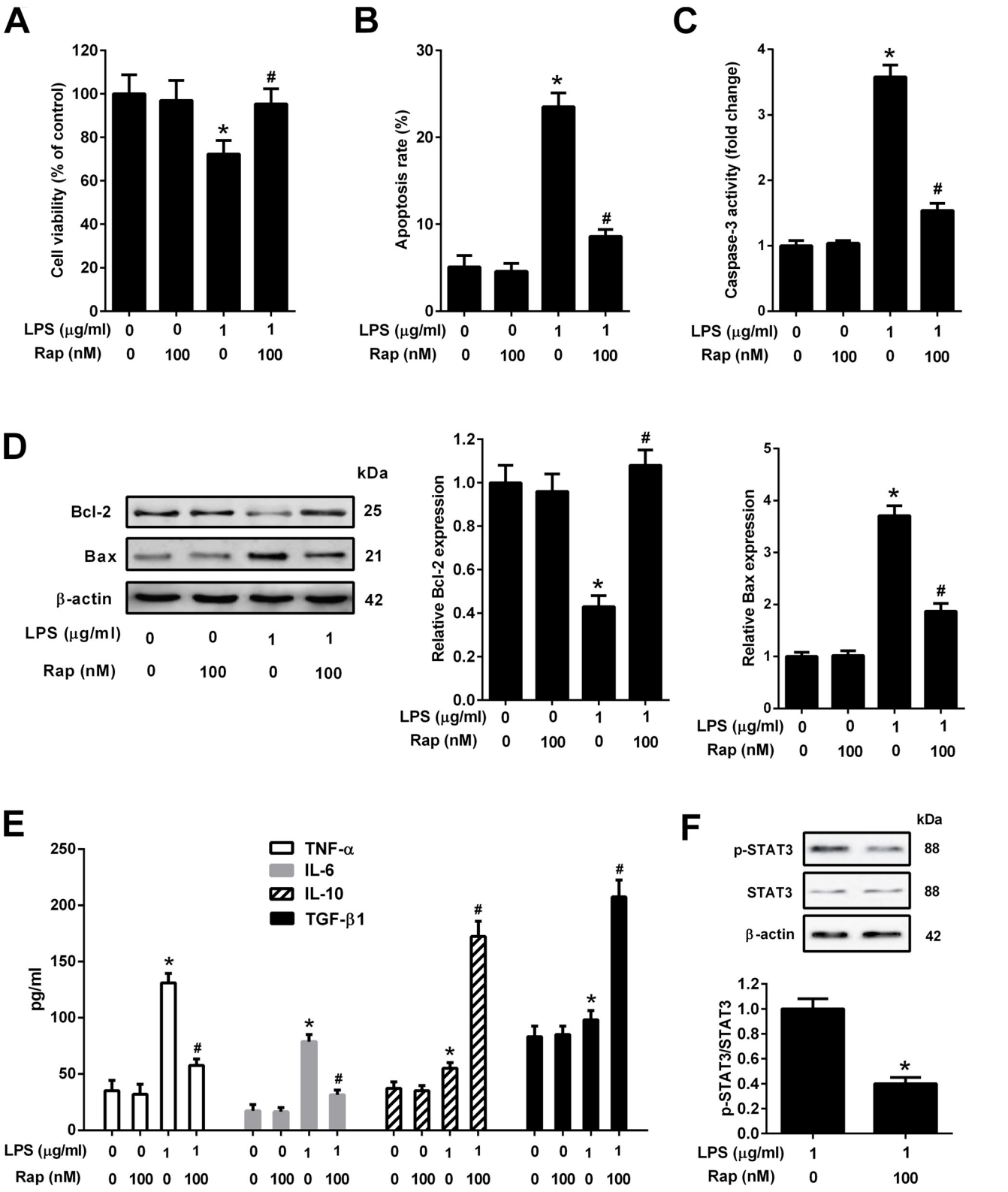

Fig. 7. Inhibition of the mTOR/STAT3 pathway restored the effects of LPS on IEC-6 cells. IEC-6 cells were treated with or without rapamycin (100 nM) and LPS (l μg/ml) for 24 h. (A) The cell viability was detected by MTT assay. $^{*}p < 0.05$ vs. control cells (without treatment of LPS and rapamycin). $^{\#}p < 0.05$ vs. cells treated with LPS only. Significance was determined by one-way ANOVA. (B) The apoptosis rate was detected by flow cytometry. $^{*}p < 0.05$ vs. control cells (without treatment of LPS and rapamycin). $^{\#}p < 0.05$ vs. cells treated with LPS only. Significance was determined by one-way ANOVA. (C) Determination of the caspase-3 activity. $^{*}p < 0.05$ vs. control cells (without treatment of LPS and rapamycin). $^{\#}p < 0.05$ vs. cells treated with LPS only. Significance was determined by one-way ANOVA. (D) The expression levels of Bcl-2 and Bax were detected by western blot. $^{*}p < 0.05$ vs. control cells (without treatment of LPS and rapamycin). $^{\#}p < 0.05$ vs. cells treated with LPS only. Significance was determined by one-way ANOVA. (E) The secretion of TNF-α, IL-6, IL-10, and TGF-β1 from IEC-6 cells were measured by ELISA. $^{*}p < 0.05$ vs. control cells (without treatment of LPS and rapamycin). $^{\#}p < 0.05$ vs. cells treated with LPS only. Significance was determined by one-way ANOVA. (F) The expression of STAT3, and p-STAT3 was detected by western blot. $^{*}p < 0.05$ vs. cells treated with LPS only. Significance was determined by Student' s *t* test. Rap, rapamycin.

as mTOR [35]. In the present study, we found that mTOR was induced by LPS and inhibited by ECH. The inhibitor of mTOR also inhibited the STAT3 expression. The results indicated that the mTOR/STAT3 pathway was involved in the effect of ECH.

In summary, the present study indicated that ECH alleviated LPS-induced cell apoptosis in rat intestine epithelial cells. The pro-inflammatory cytokine secretion induced by LPS was attenuated by ECH. The mTOR/STAT3 pathway was induced by LPS, while the induction was inhibited by ECH. The inhibitor of mTOR reversed the effect of LPS on rat intestine epithelial cells. The findings suggested that ECH attenuated inflammation and cell apoptosis induced by LPS in rat intestine epithelial cells via suppressing the mTOR/STAT3 pathway. The results indicated that ECH might be a new therapy for the treatment of IBD.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgement

None

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